

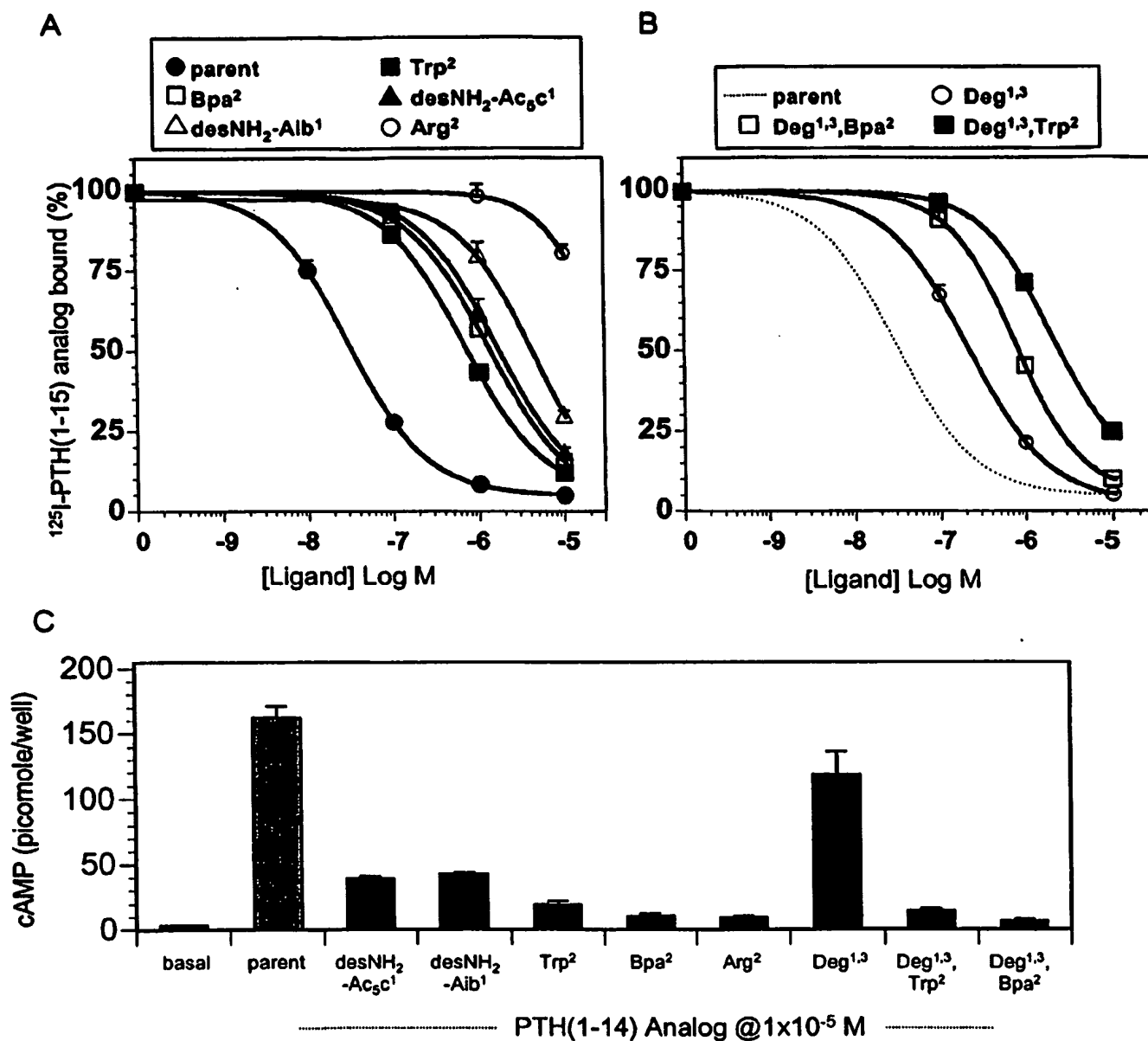
SEQ ID NO.	Peptide	Sequences
	PTH(1-14) peptides	
26	PTH(1-14)NH ₂ (native, rat)	Ala-Val-Ser-Glu-Ile-Gln-Leu-Met-His-Asn-Leu-Gly-Lys-His-NH ₂
27	[Ala, ^{3,12} ,Gln ¹⁰ ,Har ¹¹ ,Trp ¹⁴]PTH(1-14)NH ₂	Ala-Val-Ala-Glu-Ile-Gln-Leu-Met-His-Gln-Har-Ala-Lys-Trp-NH ₂
14	[Ac ₃ c ¹ ,Aib ³ ,Gln ¹⁰ ,Har ¹¹ ,Ala ¹² ,Trp ¹⁴]PTH(1-14)NH ₂	Ac ₃ c-Val-Aib-Glu-Ile-Gln-Leu-Met-His-Gln-Har-Ala-Lys-Trp-NH ₂
15	[desNH ₂ -Ac ₃ c ¹ ,Aib ³ ,Gln ¹⁰ ,Har ¹¹ ,Ala ¹² ,Trp ¹⁴]PTH(1-14)NH ₂	(desNH ₂)Ac ₃ c-Val-Aib-Glu-Ile-Gln-Leu-Met-His-Gln-Har-Ala-Lys-Trp-NH ₂
16	[desNH ₂ -Aib ³ ,Aib ³ ,Gln ¹⁰ ,Har ¹¹ ,Ala ¹² ,Trp ¹⁴]PTH(1-14)NH ₂	(desNH ₂)Aib-Val-Aib-Glu-Ile-Gln-Leu-Met-His-Gln-Har-Ala-Lys-Trp-NH ₂
17	[Ac ₃ c ¹ ,Trp ² ,Aib ³ ,Gln ¹⁰ ,Har ¹¹ ,Ala ¹² ,Trp ¹⁴]PTH(1-14)NH ₂	Ac ₃ c-Trp-Aib-Glu-Ile-Gln-Leu-Met-His-Gln-Har-Ala-Lys-Trp-NH ₂
18	[Ac ₃ c ¹ ,Bpa ² ,Aib ³ ,Gln ¹⁰ ,Har ¹¹ ,Ala ¹² ,Trp ¹⁴]PTH(1-14)NH ₂	Ac ₃ c-Bpa-Aib-Glu-Ile-Gln-Leu-Met-His-Gln-Har-Ala-Lys-Trp-NH ₂
19	[Ac ₃ c ¹ ,Arg ² ,Aib ³ ,Gln ¹⁰ ,Har ¹¹ ,Ala ¹² ,Trp ¹⁴]PTH(1-14)NH ₂	Ac ₃ c-Arg-Aib-Glu-Ile-Gln-Leu-Met-His-Gln-Har-Ala-Lys-Trp-NH ₂
20	[Deg ^{1,3} ,Gln ¹⁰ ,Har ¹¹ ,Ala ¹² ,Trp ¹⁴]PTH(1-14)NH ₂	Deg-Val-Deg-Glu-Ile-Gln-Leu-Met-His-Gln-Har-Ala-Lys-Trp-NH ₂
21	[Deg ^{1,3} ,Trp ² ,Gln ¹⁰ ,Har ¹¹ ,Ala ¹² ,Trp ¹⁴]PTH(1-14)NH ₂	Deg-Trp-Deg-Glu-Ile-Gln-Leu-Met-His-Gln-Har-Ala-Lys-Trp-NH ₂
22	[Deg ^{1,3} ,Bpa ² ,Gln ¹⁰ ,Har ¹¹ ,Ala ¹² ,Trp ¹⁴]PTH(1-14)NH ₂	Deg-Bpa-Deg-Glu-Ile-Gln-Leu-Met-His-Gln-Har-Ala-Lys-Trp-NH ₂
23	[Ac ₃ c ¹ ,Trp ² ,Aib ³ ,Nle ⁸ ,Gln ¹⁰ ,Har ¹¹ ,Ala ¹² ,Tyr ¹⁴]PTH(1-14)NH ₂	Ac ₃ c-Trp-Aib-Glu-Ile-Gln-Leu-Nle-His-Gln-Har-Ala-Lys-Tyr-NH ₂
24	[Ac ₃ c ¹ ,Bpa ² ,Aib ³ ,Nle ⁸ ,Gln ¹⁰ ,Har ¹¹ ,Ala ¹² ,Tyr ¹⁴]PTH(1-14)NH ₂	Ac ₃ c-Bpa-Aib-Glu-Ile-Gln-Leu-Nle-His-Gln-Har-Ala-Lys-Tyr-NH ₂
25	[Deg ^{1,3} ,Bpa ² ,Nle ⁸ ,Gln ¹⁰ ,Har ¹¹ ,Ala ¹² ,Trp ¹⁴ ,Arg ¹⁹ ,Tyr ²¹]PTH(1-21)NH ₂	Deg-Bpa-Deg-Glu-Ile-Gln-Leu-Nle-His-Gln-Har-Ala-Lys-Trp-Leu-Ala-Ser-Val-Arg-Arg-Tyr-NH ₂
	N-truncated peptides	
28	[Aib ³ ,Nle ⁸ ,Gln ¹⁰ ,Har ¹¹ ,Ala ¹² ,Trp ¹⁴ ,Arg ¹⁹ ,Tyr ²¹]PTH(3-21)NH ₂	Aib-Glu-Ile-Gln-Leu-Nle-His-Gln-Har-Ala-Lys-Trp-Leu-Ala-Ser-Val-Arg-Arg-Tyr-NH ₂
29	[Ile ⁵ ,Trp ²³ ,Tyr ³⁶]PTHrP(5-36)NH ₂	Ile-Gln-Leu-Leu-His-Asp-Lys-Gly-Lys-Ser-Ile-Gln-Asp-Leu-Arg-Arg-Arg-Phe-Phe-Leu-His-His-Leu-Ile-Ala-Glu-Ile-His-Thr-Ala-Glu-Tyr*-NH ₂
31	[Ile ⁵ ,Leu ¹¹ ,D-Trp ¹² ,Trp ²³ ,Tyr ³⁶]PTHrP(5-36)NH ₂	Ile-Gln-Leu-Leu-His-Asp-Leu-DTrp-Lys-Ser-Ile-Gln-Asp-Leu-Arg-Arg-Arg-Phe-Phe-Leu-His-His-Leu-Ile-Ala-Glu-Ile-His-Thr-Ala-Glu-Tyr*-NH ₂
	¹²⁵ I-PTH tracer radioligand	
32	[Aib ^{1,3} ,Nle ⁸ ,Gln ¹⁰ ,Har ¹¹ ,Ala ¹² ,Trp ¹⁴ ,Tyr ¹⁵]PTH(1-15)NH ₂	Aib-Val-Aib-Glu-Ile-Gln-Leu-Nle-His-Gln-Har-Ala-Lys-Trp-Tyr*-NH ₂

Figure 1

	Peptides	IC ₅₀		
	PTH(1-14) peptides		nM	n
##	parent	30	±7	3
##	desNH ₂ -Alb ¹	4,500	±700	4
##	desNH ₂ -AC ₅ C ¹	1,800	±100	4
##	Arg ²	25,000	±2,000	4
##	Trp ²	770	±110	4
##	Bpa ²	1,400	±200	4
##	Deg ^{1,3}	230	±50	3
##	Deg ^{1,3} ,Trp ²	2,700	±300	3
##	Deg ^{1,3} ,Bpa ²	840	±110	3
	Other peptides			
##	rPTH(1-34)	4.8	±0.8	3
##	PTHrP(5-36)	5.5	±1.0	3
##	[Aib3,M]PTH(3-21)	750	±90	3
##	[Aib1,3,M]PTH(1-21)	18	±4	3

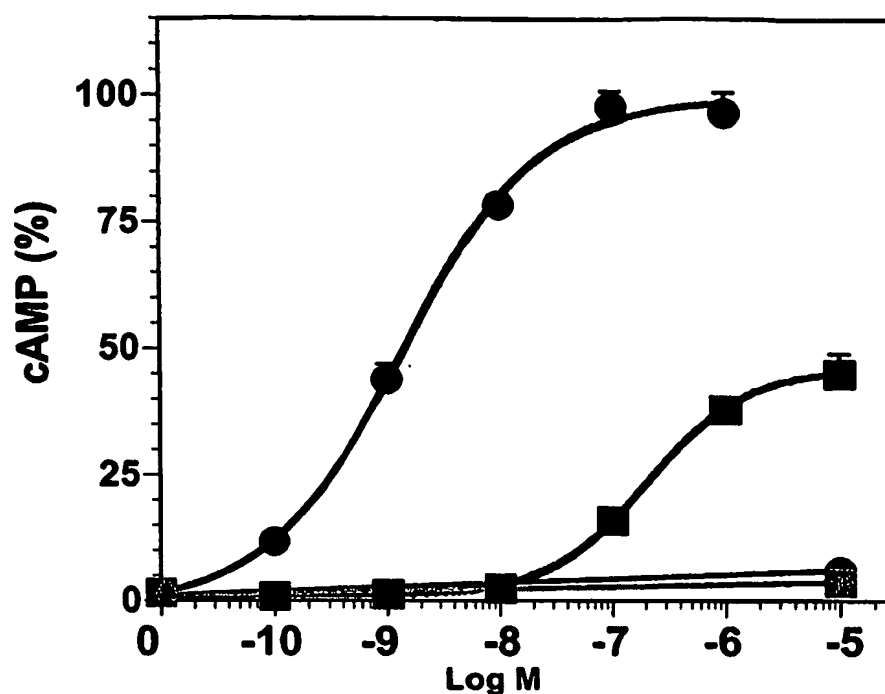
Figure 2

Figure 3



Functional Responses in HKRK-B28 Cells. Binding (A and B) and cAMP agonism/partial agonism assays (C) were performed in HKRK-B28 cells. The parent peptide was [AC5C1,Aib3,Gln10,Har11,Ala12,Trp14]PTH(1-14)NH₂ and derivatives thereof were substituted at positions 1, 2 and/or 3, as indicated. Binding assays (4h @ 15°C) were performed with ¹²⁵I-[Alb1,3,Nle8,Gln10,Har11,Ala12,Trp14,Tyr15]PTH(1-15)NH₂ tracer. cAMP assays were performed at RT for 30 min. Relative to the parent, the substituted analogs lack appreciable agonist activity.

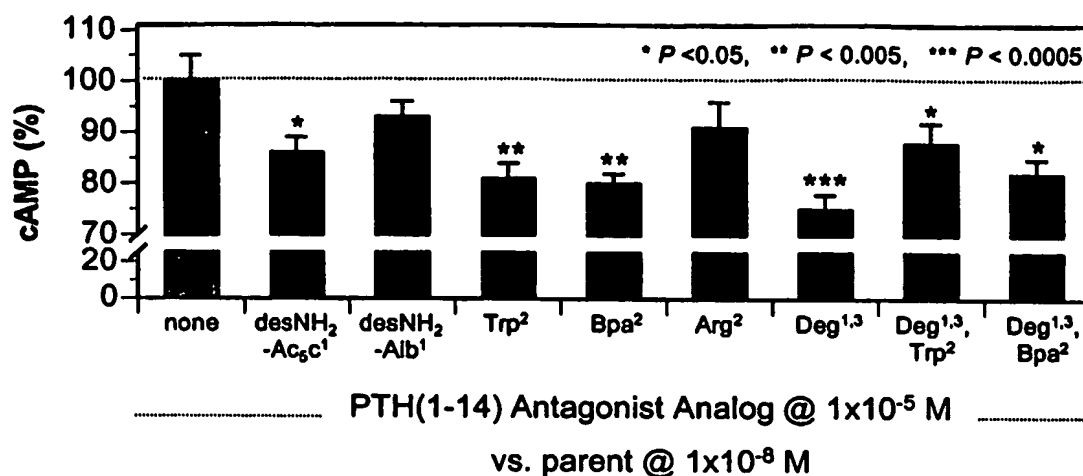
Figure 4



	<u>EC₅₀ nM</u>
● parent	1.7±0.3
■ Deg ^{1,3}	220±50
● Bpa ²	-----
■ Deg ^{1,3} ,Bpa ²	-----

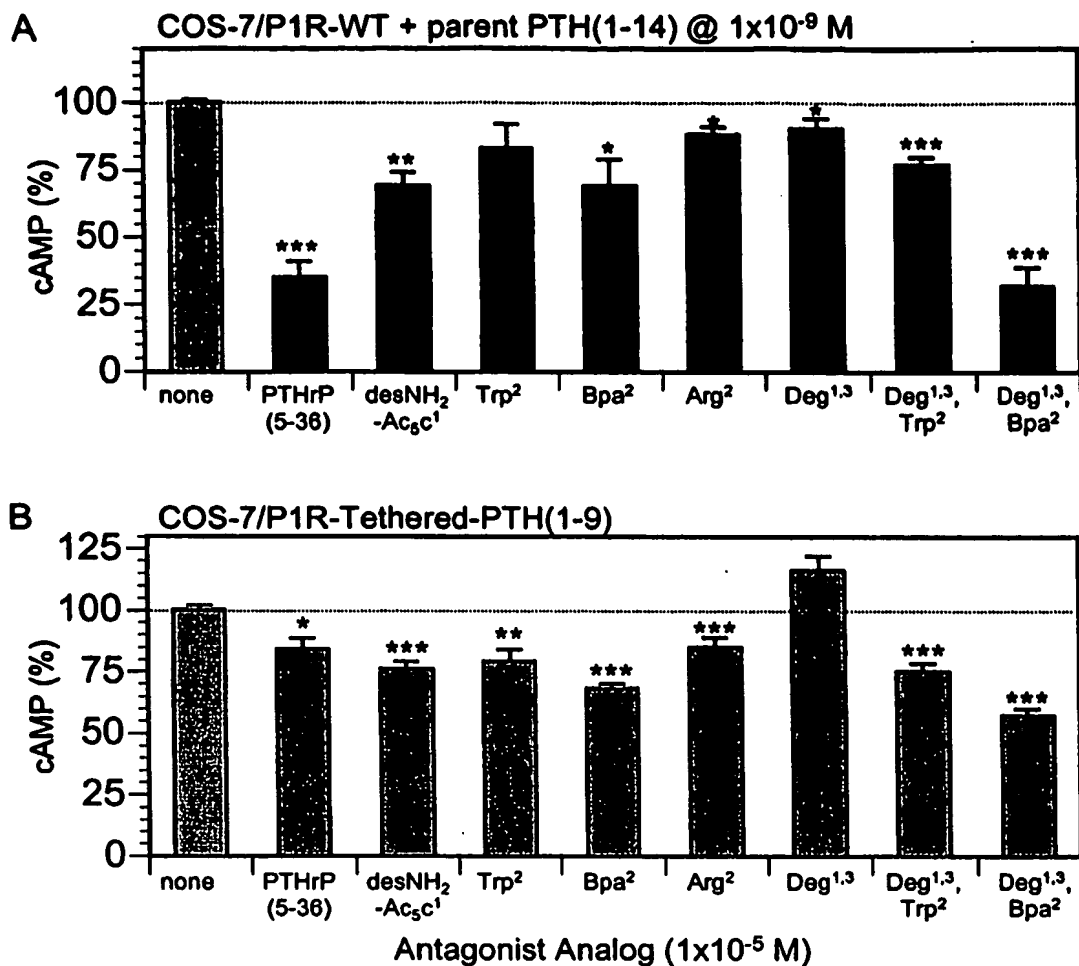
cAMP Responses in HKRK-B28 Cells. The parent peptide, [AC5C1,Aib3,Gln10,Har11,Ala12,Trp14]PTH(1-14)NH₂, and derivatives thereof substituted at positions 1, 2 and/or 3, as indicated, were assayed for cAMP agonist responses in HKRK-B28 cells. The parent peptide functions as a fully potent and efficacious agonist, the Deg^{1,3}-substituted analog is a partial agonist, and the Bpa²-substituted analogs lack agonist activity.

Figure 5



Antagonism Assays in HK-RK-B28 Cells. cAMP antagonism assays were performed in HKRK-B28 cells. Cells were treated with the J domain-selective agonist, [AC5C1,Aib3,Gln10,Har11,Ala12,Trp14]PTH(1-14)NH₂ (parent) at 10 nM, either alone (none) or with a candidate antagonist peptide (10 μ M), which was a derivative of the parent PTH(1-14) peptide substituted at positions 1, 2 and/or 3, as indicated. Asterisks indicate significant reductions in cAMP levels, as compared to cells not treated with antagonist (none).

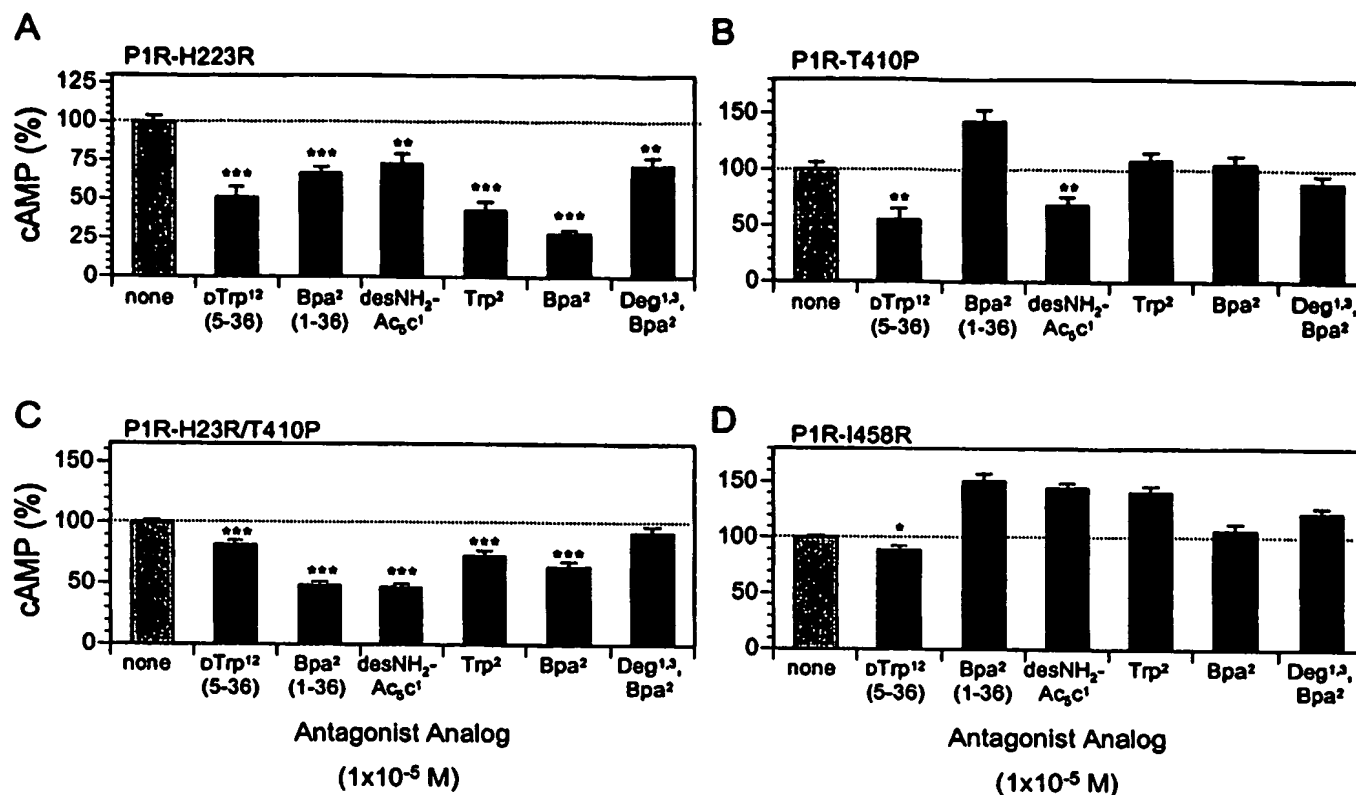
Figure 6



* $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$

Antagonism Assays in COS-7 Cells. cAMP antagonism assays were performed in COS-7 cells transfected with the wild-type P1R (A), or a constitutively active P1R derivative having the first 9 residues of PTH tethered to TM1 of the P1R and in place of the P1R N-terminal domain (inset, B). In A, cells were treated with the J domain-selective agonist, [AC5C1,Aib3,Gln10,Har11,Ala12,Trp14]PTH(1-14)NH₂ (parent) at 1 nM, alone (none) or with a candidate antagonist peptide (10 μ M), which was a derivative of the parent PTH(1-14) peptide substituted at positions 1, 2 and/or 3, as indicated, or [I5,W23,Y36]PTHrP(5-36) analog. Asterisks indicate significant reductions in cAMP levels, as compared to cells not treated with antagonist (none).

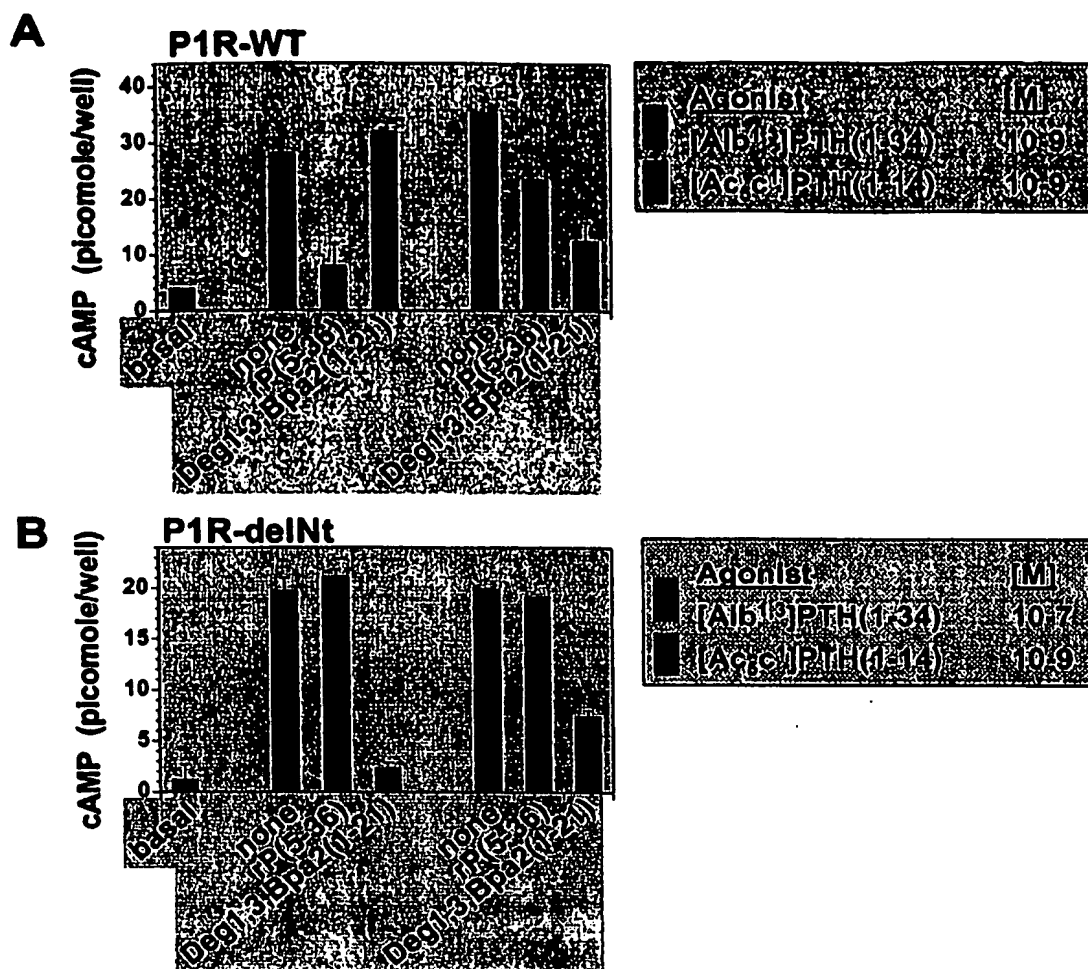
Figure 7



* $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$

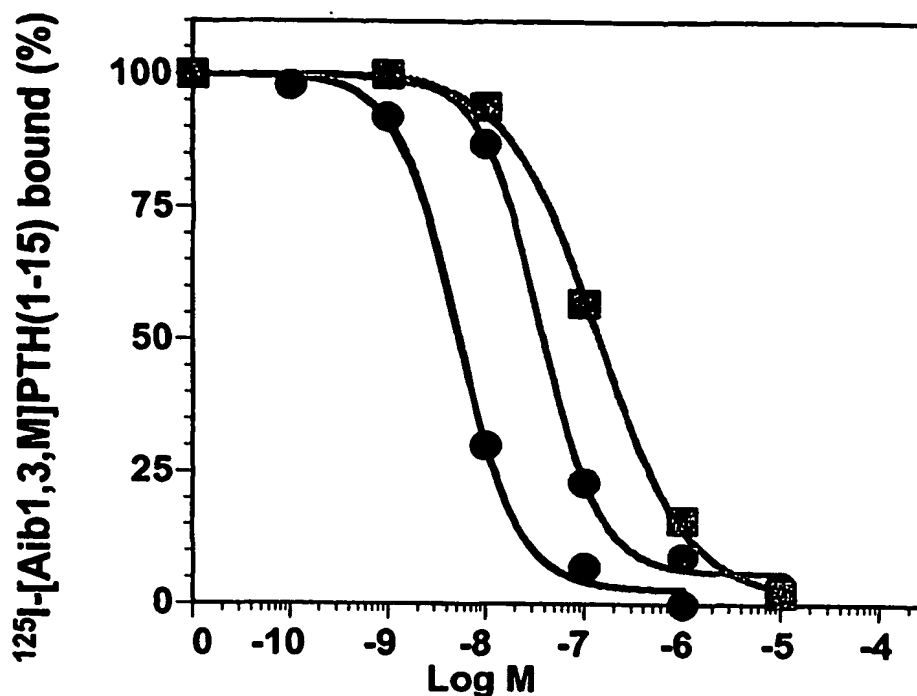
Inverse Agonist Responses in COS-7 Cells. COS-7 cells were transfected with the constitutively active P1Rs: P1R-H223R (A), P1R-T410P (B), P1R-H223R/T410P (C), or P1R-I458R (D) and then were incubated (30 min@R.T.) either in the absence of peptide (none) or in the presence of the indicated antagonist/inverse agonist peptide (10 μ M), and cAMP was measured by RIA. Asterisks indicate significant reductions in cAMP levels, compared to untreated cells (none).

Figure 8



"N" versus "J" Domain selectivity of P1R Antagonists in COS-7 Cells. cAMP antagonism assays were performed in COS-7 cells transfected with the wild-type P1R (A), or a P1R derivative (P1R-delNt) having most (residues 24-181) of the P1R N domain deleted (B). Cells were treated with the agonist [Aib1,3,Tyr34]hPTH(1-34)NH₂ ([Aib1,3]PTH(1-34)), which utilizes both N and J domains for affinity/potency, or with [AC5C1,Aib3,Gln10,His11,Ala12,Trp14]PTH(1-14)NH₂ ([Ac5c1]PTH(1-14)), which uses only the J domain for affinity/potency, at the concentrations indicated in the key, so as to elicit half-maximum cAMP responses in the absence of antagonist (none). The analogs PTHrP(5-36) and Deg1,3,Bpa2-PTH(1-21) were added at 1×10^{-5} M, as indicated. On the WT receptor, PTHrP(5-36) antagonizes PTH(1-34) analog more effectively than does Deg1,3,Bpa2-PTH(1-21), but the PTH(1-21) analog antagonizes PTH(1-14), more effectively than does PTHrP(5-36). On P1R-delNt, Deg1,3,Bpa2-PTH(1-21) antagonizes either agonist, whereas PTHrP(5-36) lacks antagonist capability. Thus, PTHrP(5-36) is an N domain-selective antagonist, whereas Deg1,3,Bpa2-PTH(1-21) is a J domain-selective antagonist. The analog Deg1,3,Bpa2-PTH(1-14) behaved similarly in these assays to Deg1,3,Bpa2-PTH(1-21).

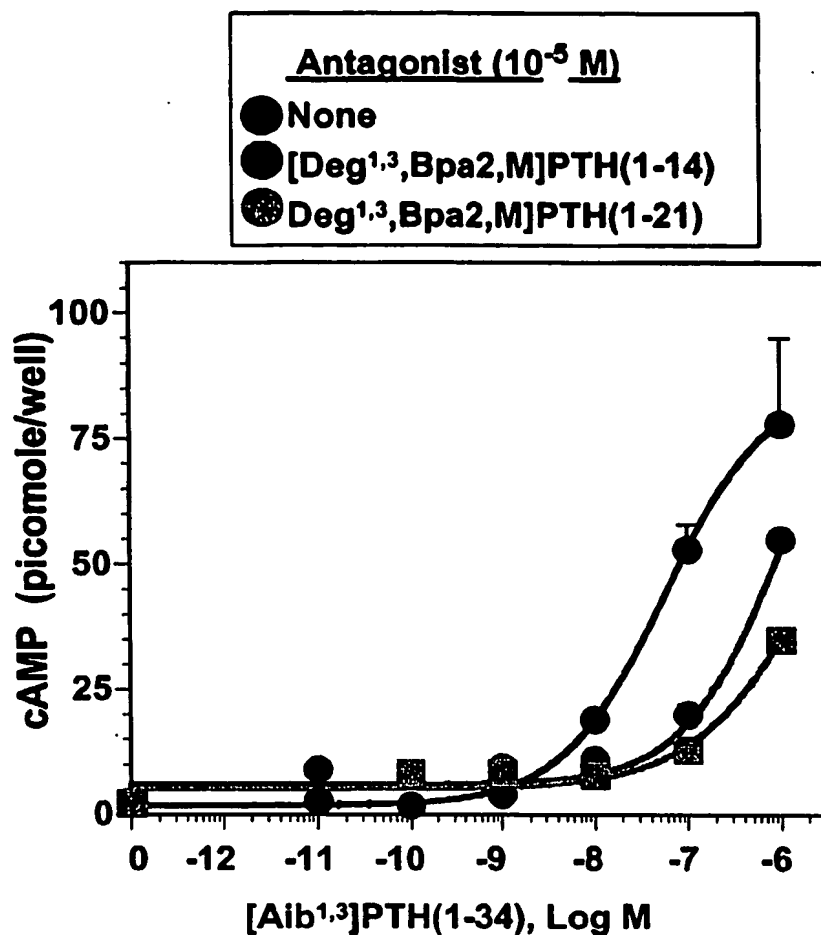
Figure 9



	<u>IC₅₀(nM)</u>
● PTH(1-34)	2
● PTHrP(5-36)	50
■ [Deg ^{1,3} ,Bpa ² ,Y ¹⁵ ,M]PTH(1-21)	150

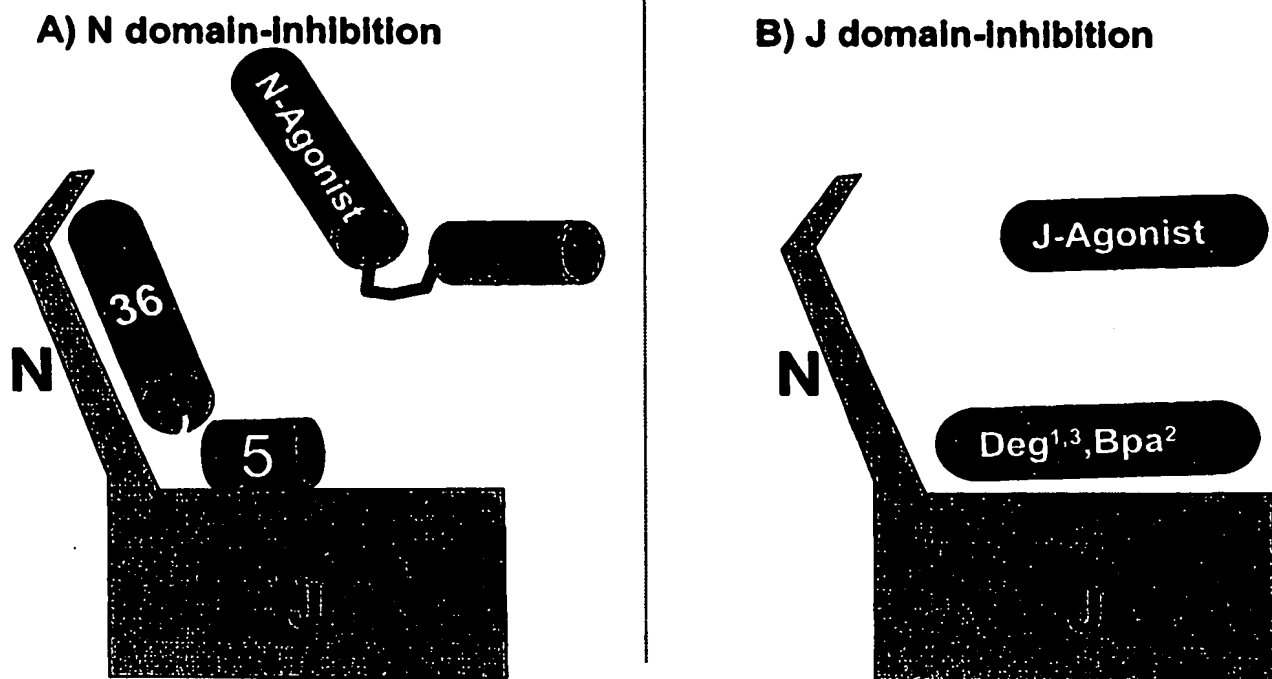
Competition Binding Assays in HKRK-B7 Cells. Binding assays were performed in HKRK-B7 cells, which express the wild-type hP1R, using ¹²⁵I-[Aib1,3,Nle8,Gln10,Har11,Ala12,Trp14,Tyr15]PTH(1-15)NH₂ as a tracer radioligand and the indicated unlabeled peptides as competitors. PTH(1-34) is [Tyr34]hPTH(1-34)NH₂.

Figure 10



Competitive Antagonism on P1R-deINt. COS-7 cells transfected with P1R-deINt were stimulated with varying concentrations of the agonist [Aib^{1,3},Tyr³⁴]hPTH(1-34)NH₂ ([Aib^{1,3}]PTH(1-34)), either in the absence of antagonist (green circles) or in the presence of an antagonist, [Deg^{1,3},Bpa2,M]PTH(1-14) (red circles) or [Deg^{1,3},Bpa2,M]PTH(1-21) (yellow squares) each at 1×10^{-5} M, as indicated in the figure key. Each antagonist causes a parallel, right-ward shift in the agonist dose-response curve, which is consistent with a competitive mechanism of inhibition.

Figure 11



Two Modes of Competitive Inhibition at the P1R. Two modes of antagonism are now recognized at the P1R. N domain inhibition (A) is utilized by most conventional P1R antagonists, such as PTHrP(5-36) and PTHrP(7-34) analogs, and is based on the derivation of binding energy primarily from interactions between the (21-34) region of the ligand and the P1R N domain. This mechanism is effective for inhibition of N-domain-dependent agonists, such as PTH(1-34), but not for N domain-independent agonists, such as PTH(1-14). J domain inhibition (B) is utilized by the novel analogs described herein, and is based on the derivation of binding energy primarily or wholly from interactions between the (1-20) region of the ligand and the J domain of the P1R. This mechanism is effective for inhibition of J-domain-dependent agonists, such as PTH(1-14) analogs, but not for N domain-dependent agonists, such as PTH(1-34). A J domain-selective antagonists would be useful for characterizing small-molecules that act as PTH mimetics, since such molecules are likely to bind to the J domain.

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